BSPS PROGRAM (ESI-MASS SPECTROMETRY)
BIOLOGICAL SAMPLE DATA ANALYSIS; DISRUPTION OF BACTERIA SPORES

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ABSTRACT

The various biological processing technologies and biological identification approaches are essential for support of the mission to develop and demonstrate an advanced Biological Sample Preparation System. The BSPS will be used in conjunction with ESI-mass spectrometry for biological point detection and identification purposes. This report discusses the design and testing of a model for biological sample identification using mass spectrometry and protein sequence information available for various organisms. This is based on nature of various organisms and their protein sequence entries available, including experimentally verified posttranslational modifications. It is also based on significance based testing. Tested the model for biological sample data analysis including positive and negative ion MALDI TOF mass spectra for *protein extracts of H. pylori (26695)* samples (reported in literature). This model may be used in conjunction with pattern recognition algorithms for Bio-sample ID through sample processing and ESI mass spectrometry. Protein biomarkers (LC-ESI as well as MALDI TOF MS) data available for some environmental bacteria samples show variability in the mass tables obtained at different growth times for the bacteria. In one comparison, common protein masses, obtained from sets of any two-growth times, were 20% of those obtained over the entire period. This supports the integration of two models for Bio-sample ID, as well as various sets of Bio-sample data as mentioned above. The decision making of the various biological sample protein mass spectral data may be made with the help of relational tools and by the use of software. Disruption of BG spores by the use of sonication or/and beads. is evaluated. Results indicate more significant protein like peaks, from the BG spore protein extracts obtained by the use of Beads Beater. Proteins were separated with reverse phase HPLC columns and detected by UV detector.

INTRODUCTION

BSPS program comes under the contamination avoidance programs for Chemical and Biological defense. As stated in the section 3. 1, of the Military critical Technology List , contamination avoidance deals with providing real time detection and identification of toxic agents. Detectors used at designated locations are called point detectors. The technology focus is on detection, warning and identification across the spectrum of CB agents as well as on the integration of CB detectors into various platforms. Detection technologies must be capable of sensing and mapping large areas of non-volatile liquid chemical agent contamination. The various biological processing technologies, and identification approaches are essential for support of the mission to develop and demonstrate an advanced Biological Sample Preparation System. The BSPS will be used in conjunction with ESI- mass spectrometry for biological sample detection and identification purposes. It should have short response time with a low rate of false returns and meet appropriate size, weight, and power requirements. **This report will be limited to discussion of technologies of sonication and/or beads for the disruption of bacteria spores. This report also deals with various approaches for detection and identification of bacteria by the use of BSPS and ESI-MS. That includes the use of available protein sequences for various organisms in the SWISS PROT as well as use of mass tables**.

One of the requirements for various technologies used for detection of biological samples is the **disruption of the bacterial spores**. Sonication is a widely practiced method to rapidly disrupt a variety of cell types[18-21]. BG spores are part of a panel of standards used for evaluating the performance of new instrumentation for bioagent detection. The effective lysis of bacteria spores is very important for the success of the BSPS (MS) for detection and identification of biological samples. The mechanism in which sonication forces disrupt cells has been proposed to be gaseous cavitation. [18-21]. In this process, pockets of air form from the dissolved gases in a solution and then rapidly collapse to a portion of the original size, creat-

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ing high pressure and temperature microenvironments that are damaging to cells. *Bacillus* spores, unlike cells, are very resistant to these damaging forces. Use of extended time for sonication may result in increase in temperature. Spore heat resistance is reduced when combined ultrasonic and heat treatments are applied[23]. Chill conditions may pose problems in logistic considerations. Berger and Marr reported that *Bacillus* spores required 1 h of continuous exposure to sonication before significant spore disruption could be measured. However, this disruption time could be significantly reduced by placing glass beads in the suspension. The beads provide more surface area for air pocket formation, resulting in an increase in the number of cavitations events during sonication treatment. [19-21]. There are various reports describing the use of beads in conjunction of sonication to extract the DNA from bacteria spores. Micro fluidic devices have been developed, which use beads in conjunction with sonication, for processing the bacterial spore samples[22.] Further details regarding these micro fluidic devices for the biological sample processing and other related information is discussed in the ECBC Technical report (Under publication.)

There are various MS approaches for rapid **detection, characterization / identification of** microorganisms. Following are the approaches, based on the **detection** of unique biomarkers from the biological samples. (1). Cell wall lipids as bacterial biomarkers. [1, 2.] (2) Proteins expressed in microorganisms as biomarkers. Organism identification and classification has been attempted by using the mass spectra of protein extracts from biological samples, obtained on matrix assisted laser adsorption/ionization (MALDI) time of flight (TOF) instruments as well as on electro spray ionization instruments. [3-7] The fingerprint protein profile for different biological samples, i. e. The pattern of observed mass spectral peaks typically in the mass range of 4-15 kDa is generally used for identification purposes. The critical requirement is mass spectral reproducibility. However, it is known that there are a number of variables affecting the mass spectra of the protein extracts from these biological samples. There may be variability of proteinous biomarkers detected from the biological samples due to bacterial culture growth time[8] and sample pretreatment[9]. · Also the fingerprint mass spectral database of microorganisms may also be shaped by both the ionization technique. ECBC, with University collaboration, has been involved in generating protein mass tables that are tailored for bacterial identification. So for we have mass tables *for Escherichia coli, Bacillus subtilis, C. friendii, B. magaterium.* After biological sample processing, for various bacteria samples, proteinous biomarkers are detected by MALDI TOF or ESI Mass spectrometry of the protein extracts. Protein fractionation has been achieved by HPLC. [10-12] Some of these proteinous mass tables for various organisms are generated as a function of bacterial culture incubation time.

An alternative approach, proposed by Fenselau and co-workers, involves matching the mass spectra obtained from the protein extracts of biological samples with the protein sequences information for these organisms, available on the public databases.[13-16] Demirov and others (2001) reported the identification of Helicobacter pylori from a list of bacteria whose individual proteome size in the mass range from 4 to 20 kDa exceeds about 200 entries. [15] Post translational modifications of ribosomal proteins, which involve N-terminal methionine cleavage, was also taken into account. The statistical significance testing showed more usefulness for application to bacteria with low proteome size. In fact a simple application of the same procedure fails if the protein sequences are downloaded later, when the proteome size changes.

Generalizations/rules for the post translational modifications of proteins, applicable to bacteria, may be helpful in correcting the available protein sequences for various bacteria, available in SWISS PROT. These may also be used in making corrections to the proteome, for the identification purpose. As introduced in the methods of enzymology, usage of the term post translational goes back to the original usage of the translational as a broad description of the entire process by which the polymer of the three-letter code in the mRNA is translated. There is extensive review of post transnational modifications of proteins by Finn Wold(1981)[24], given as in vivo chemical modifications of proteins. A brief summary of this review, applicable to bacteria systems, along with appropriate specific sources for the individual cases of post translational modifications is described in the ECBC technical report (Under publication). There are some rules and generalizations, applicable to post translational modifications of proteins in bacteria. Some of these are reported by Wilkins and others (1999)[25].

Acetylation, b-methyl thiolation, biotin, bromination, carbamylation, deamidation, methylation, glucosylation, lipoyl, phosphorylation,, pyridoxal phosphate, phosphopantttheine, pyrrolidone carboxylic acid. Rules were constructed by examining 5153 incidences of posttranslational modifications documented in

SWISS PROT, that includes the modifications listed above. [25] These consider which amino acids can carry a modification, whether modifications occurs on N- terminal, C-terminal or internal amino acids, and the type of organisms on which the modifications can be found.

The present BSPS report discusses the design for a model system for the identification of bacteria, by the use of protein biomarkers for various biological samples detected by MALDI TOF or ESI mass spectrometry, and the molecular weights for various protein sequences available on SWISS PROT. This uses the correction or weighted correction to the various proteomes for bacteria, for avoiding false matches. This also has been used in conjunction with significance testing, in some cases. Corrections to the proteome may deal with proteins sequence and also on the nature of biological species related protein sequence entries, that includes, partial protein sequences, and experimentally obtained post transnational modifications of proteins, for various bacteria. This BSPS also gives a brief review of certain generalities in the area of post translational modifications of proteins. That will be used for further modifications of the design for model system, for the identification of bacteria, by the use of biological sample processing, mass spectrometry, and use of protein sequences and their molecular weights reported in SWISSPROT. The present BSPS report uses this model system for identification of certain bacterial samples. The protein biomarkers used had been detected through MALDI TOF or ESI mass spectrometry. This also reviews the variability obtained in the protein biomarker data obtained as a function of different growth time for cultures of various environmental bacteria, such as *Escherichia coli, Bacillus subtilis*. This report applies the protein mass tables available for Bacillus subtilis to compare the correlation/ranking of the MALDI TOF mass spectra of the protein extracts for Bacillus subtilis and B. magaterium, for the purpose of their identification. The protein biomarkers from biological samples, were detected by ESI-MS or MALDI TOF mass spectrometry. This report also addresses technologies for the biological sample processing, that deals with disruption of bacteria, in particular, by the use of sonication or /and beads. It addresses the problem of lysis of BG spores by making comparison of BG spore lysis with sonication and Or Beads. It reports on the results of the preliminary experiments conducted for the disruption of BG spores, by the use of sonication and /or Beads.

EXPERIMENTAL

This section gives the materials used as well as methodologies used, such as sample preparation, mass spectrometry, as reported in literature, corresponding to the mass spectra used for detection of protein biomarkers in various biological samples. This also reports the search of protein biomarkers from the public databases.

H. pylori, strain 26695, was obtained from ATCC (Manassas, VA). The details of experimental conditions used for growing H. Pylori cultures , have been described. [17]

Sample preparation used for conducting MALDI TOF mass spectrometry for the H. Pylori samples used have been described by Demirev and others(1999)[13.] The B. subtilis(strain168, ATCC 23857) and E. coli (ATCC11775), were grown in House and the details of the procedure are also described by the same authors. The *B. subtilis (9392) and E. coli(47076) and E. coli(9637), B. megatarium, and C. freundii,* used by Liang Li and others, were grown in House, or obtained through ECBC. Solvent delivery and separations were performed on an Agilent (Palo Alto, CA) HP1100 HPLC system[17]. The biological sample processing, sample preparation procedures and various materials used for chromatography columns, and other parameters used for sample preparation procedure for MALDI TOF or ESI mass spectrometry for these studies have been described. [11-12]. The HPLC effluent was analyzed with a HP 1100 MSD quadrupole mass spectrometer (Agilent). The *m/z* range from 500 to 3000 was scanned in 1. 90 s, and the ions were detected with a high-energy dynode detector. Control of both the HPLC and MS systems was accomplished with HP ChemStation software. Details of experimental condition, for ESI-MS, are described by Liang, Li. And others (2002)[17]. Access to SWISS PROT was obtained by www. expasy. ch/

Following are some of the experimental details for the preliminary experiments conducted for comparing the disruption of BG spores by the use of sonication and/or beads; Approximately 50 mg/ml of the BG spore material (8. 5x10exp. 10 cfu/mg) was used for these experiments. BG spore were suspended in 5% acetic acid/5% acetonitrile (0. 1% TFA). 1mm silica/zirconia beads were used for the experiments. Reverse phase C8 column (Vydac) was used for separations of proteins. Proteins were detected by UV detector (214 nm). HPLC system used was from Waters (Waters 2690). Gradient mode used various compositions of water/Acetonitrile (0. 1%TFA) as the mobil phase. More details for this will be given in ECBC technical report (To be published).

RESULTS AND DISCUSSION

1. MODEL SYSTEM FOR THE IDENTIFICATION OF BACTERIA :

Following is the design of the scheme/ used for identification of bacteria, by biological Sample processing and mass spectrometry. The molecular weights for the protein biomarkers from biological samples, detected by mass spectrometry are matched with the molecular weights of the protein sequences for the various bacteria, available on SWISS Prot. Scheme has been developed by analysis of the protein sequences for various bacteria as given in the SWISS PROT. The various steps of this scheme are given as follows.

- Experimentally find the molecular weight of proteinous biomarkers by sample processing and mass spectrometry ,of the protein extracts.
- Find the total number of protein sequences, for appropriate bacteria, available in SWISS PROT, with molecular weights between 4000 and 20000, Daltons.
- Match the molecular weight of proteinous biomarkers with the molecular weights corresponding to the protein sequences available in SWISS PROT.
- Find the total number of protein sequences, which have been experimentally found to be post translationally modified in various bacteria.
- Correct the molecular weight for such protein sequence, for the appropriate modification.
- Repeat matching again between experimental mass spectra and the corrected protein proteomes.
- Correct the proteome for any common protein sequence molecular weights (This has not been done in this study, but will be tried in future).
- Correct the proteome size for various bacteria, for the various post translational modifications of proteins found through application of rules and generalities. Make corrections to the appropriate protein sequences and then repeat matching the experimentally found molecular weight of protein biomarkers for a certain biological sample. (This has not been used in this report, but may be used in future).
- Find the various protein sequence entries in SWISS PROT, which are less likely to be matched with the protein molecular weights for intact proteins, found on a mass spectrometer. Example of such will be a partial protein sequence in the swiss prot.
- Find the weight facter for the protein sequences, which might have shown a less probable as a 100% weight, for one match. This may be due to different protein entries given for various bacteria, by different researchers. A range of weight factors has been applied to test the effectiveness of this approach.

  1. N = Total number of protein sequences with molecular weight between 4000 and 20000 Daltons in the SWISS PROT= N. This is also described as the proteome size.
  2. C = Total number of corrections for the proteins in SWISS PROT protein entries 'N', corresponding to the particular bacteria. (This is the sum total of different type of corrections for the protein sequence entries)
  3. K = Total number of the proteinous biomarkers corresponding to a particular organism detected by biological sample processing and mass spectrometry.
  4. K1=Let the total number of matches obtained by matching the molecular weight of the proteinous biomarkers with the protein sequences reported in the SWISS prot.
  5. C1= Let the total number of corrections for the protein biomarkers matches with protein sequences in SWISS PROT.
  6. S1= Let the significance calculated with respect to the original proteome size 'N' and original number of number of matches 'K'. $=S1^{15}$
  7. S2 = Let the significance calculated on the basis of corrected proteome size N1, and the corrected number of matches be= S2.
  8. N1= N-C –C1 (N1 is the corrected proteome size)
  K2=K1-C1.
  9. K2= Corrected matched corresponding to mass spectrometry molecular weight for biomarkers with protein sequences in Swiss prot.
  10. Rank 1 for bacteria implies that it stands the highest correlation to be the one in the biological sample under investigation by sample processing and mass spectrometry.

11. Probability of bacteria, which stands the highest chance from the calculation of significance = 1-S where 'S' is the calculated significance for a particular situation.

2. CHARACTERIZATION OF VARIOUS BACTERIA, BY THE USE OF THE ABOVE DESIGN:

2. 1 CASE I

CHARACTERIZATION OF H. PYLORI

- Proteinous Biomarkers of ***H. Pylori*** samples are detected by MALDI TOF Mass Spectrmetry[15]
- Positive and negative ion mass spectra was obtained on KOMPACT MALDI 4 , Time of Flight instrument in a linear mode at +/-18Kv nominal accelerating voltage.

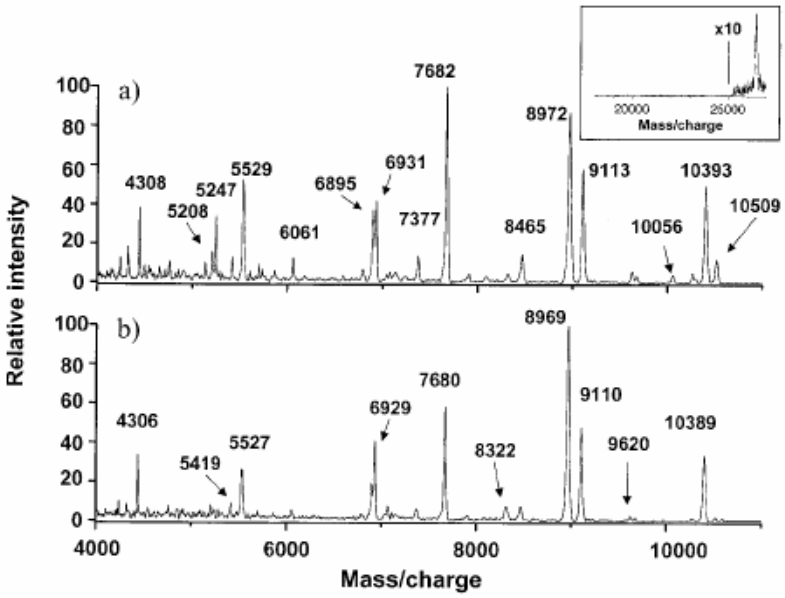


Figure 1. MALDI-TOF mass spectra from intact *H. pylori* in the *m/z* range 4000 to 11 000 in (a) positive and (b) negative ion modes. Inset: extended *m/z* range with the peak attributed to 26.4 kDa urease α-subunit protein.

TABLE 1

Matching of protein biomarkers of ***H. pylori*** protein extracts with molecular weights of protein sequences available in SWISS PROT.

NOTE: Most of the bacteria given in the tables below have their completion in genome sequence study. But this also uses those bacteria with incomplete study of their genomes sequences as well as proteomes.

Organism	N	K	K1	S1	S2
Mycobacterium tuberculosis	1655	25	5	**2. 35E-05**	**0. 089086**
Bacillus subtilis	1537	25	***16***	**7. 14E-06**	**5E-04**
Streptococcus agalactiae	1232	27	4	**4. 94E-09**	**0. 312384**
Pseudomonas putida	1787	27	15	**5. 6E-06**	**0. 00062**
Rhizobium meliloti	1834	27	9	**8. 76E-06**	**0. 021164**
Shigella flexneri	1781	28	12	**1. 24E-06**	**0. 015789**
Streptomyces avermitilis	1944	29	6	**1. 43E-06**	**0. 384713**
Bacillus cereus	2505	30	8	**3. 55E-05**	**0. 302712**
Xylella fastidiosa	1843	30	9	**1. 04E-07**	**0. 017449**

Bacillus anthracis	2243	32	8	**2. 31E-07**	**0. 249545**
Synechococcus sp	1486	32	10	**1. 88E-11**	**0. 003603**
Staphylococcus aureus	2177	35	13	**4. 96E-10**	**0. 002289**
Streptococcus pyogenes	2007	35	9	**6. 39E-11**	**0. 007282**
Neisseria meningitidis	1605	37	5	**1. 11E-15**	**0. 306302**
Vibrio vulnificus	2898	**42**	6	**4. 58E-14**	**0. 955168**
Helicobacter pylori	2370	**42**	***21***	**8E-17**	**1E-09**
Escherichia coli	6411	**92**	15	**0**	**0. 851752**

RESULTS**: H. Pylori** (H. pylori ranks Ist, as seen above in column, K1 and S2)

1. Application of Simple statistical correction give very poor results. (S1)

2. Results are better obtained after making corrections to the proteome as outlined in the scheme, above.

2. 2. CASE 2.

CHARACTERIZATION OF *B. SUBTILIS* (Strain 168 ATCC NO. 23857)

Proteinous Biomarkers of ***B. subtilis,* samples** are detected by MALDI TOF Mass Spectrmetry[13]. Kompact MALDI 4 TOF instrument (Kratos Analytical Instruments, U. K.)[13].

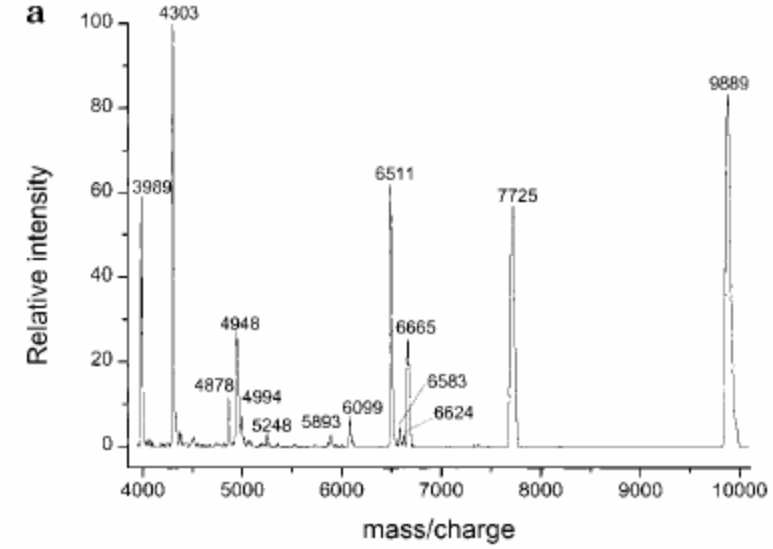


Figure 2. Positive ion MALDI spectra from B. subtilis(8 h growth time), matrix SA[13]

TABLE 2

Matching of protein biomarkers of ***Bacillus subtilis*** protein extracts with molecular weights of protein sequences available in SWISS PROT.

Organism	**N**	**K1**	**K2**
Bacillus subtilis	1537	**14**	**7**
Escherichia coli	6431	**43**	**5**
Synechococcus sp	1486	14	5
Mycobacterium tuberculosis	1655	16	2
Helicobacter pylori	2398	2	2
Vibrio cholerae	1548	9	2
Streptococcus pneumoniae	1881	14	1
Pseudomonas aeruginosa.	1876	8	3
Mycobacterium leprae	955	5	5
Streptococcus pyogenes.	2007	5	5

Yersinia pestis	1812	9	2

RESULTS : ***Bacillus subtilis*** (Bacillus subtilis ranks Ist, as seen above in column K2)

2. 3. CASE 3.

CHARACTERIZATION OF *B. MEGATERIUM:*

- Proteinous Biomarkers of ***B.*** **MEGATERIUM samples** are detected by On-Line LC/ESI-MS[17] of the protein extracts.
- HP 1100 MSD quadrupole mass spectrometer (Agilent). The *m*/*z* range from 500 to 3000[17,] was scanned in 1. 90 s, and the ions were detected with a high-energy dynode detector[17].

TABLE 3

$(M+H)^+$ from B. megetarium extract by On-line LC/ESI-MS[17] (Daltons)

3047. 5	**3186. 9**	**4379. 2**	**4741. 1**	**4816. 3**	**6262**	**6276**	**6316**	**6336**	**6352**
6389	**6393**	**6449**	**6578**	**6897**	**7111**	**7157**	**7280**	**7425**	**7451**
7467	**7519**	**7649**	**7711**	**9334**	**9351**	**9620**	**9694**	**9747**	**9754**
9768	**9829**	**9884**	**10026**	**10045**	**10453**	**10696**	**11063**	**11538**	**11612**
11726	**12046**	**12120**	**12408**						

TABLE 4

Matching of protein biomarkers of **B. MEGATERIUM** protein extracts, with molecular weights of protein sequences available in SWISS PROT.

Organism	**N**	**K1**	**K2**	**S1**	**S2**
Streptomyces coelicolor	2174	40	9	2. 72985E-15	0. 212285658
Borrelia burgdorferi	856	18	6	2. 48174E-05	0. 005154691
Bacillus subtilis	1537	25	13	7. 13993E-06	0. 012380472
Bacillus anthracis	2244	40	6	7. 22065E-15	0. 571248466
Vibrio cholerae	1548	34	10	6. 61617E-13	0. 009583315
Escherichia coli	6411	121	27	0	0. 00448299
Synechococcus sp	1486	35	10	2. 01713E-14	0. 003603358
Salmonella typhi	2758	45	17	0	0. 08465888
Prochlorococcus marinus	2123	41	20	6. 167E-17	0. 000691805
Bacillus Megaterium	99	4	**4**	0. 008649207	**0. 000657185**

RESULTS : ***Bacillus Megaterium (*** **Bacillus Megaterium ranks Ist, as seen in column S2)**

Comments:

1. Very few protein sequences for these organisms are available in SWISS PROT.
2. Application of statistical corrections directly, gives very poor results (S1)
3. Application of corrections to the proteome as outlined in the scheme, and then application of correction due to small proteome size, gives very good results.

2.4 CASE 4.

- **CHARACTERIZATION OF *Escherichia coli(ATCC N0. 11775)***
- Proteinous Biomarkers of **E. coli (ATCC 9637) samples** are detected by On-Line LC/ESI-MS[10], of the protein extracts.
- HP 1100 MSD quadrupole mass spectrometer (Agilent)[10]

Total ion chromatogram of ATCC 9637 TFA extract (Time vs. intensity of ions)

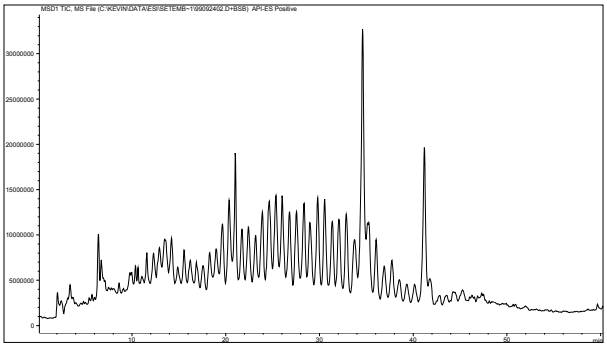


TABLE 5

Molecular weight for $(M+H)^+$ in Daltons

1087. 3	2139. 0	3624. 7	6254. 9	7781. 6	9264. 5	10459. 1
1161. 3	2375. 3	3793. 1	6315. 7	7855. 4	9518. 2	11224. 0
1194. 3	2416. 2	4480. 8	6330. 3	9064. 3	9536. 3	11297. 0
1215. 5	2431. 5	5036. 7	7140. 0	9191. 7	9610. 0	11781. 5
1235. 3	2504. 9	5096. 7	7272. 1	9209. 1	9684. 0	13093. 9
2008. 0	2735. 9	5170. 8	7274. 2	9226. 4	9740. 2	15693. 3
2123. 0	3509. 6	5550. 1	7707. 3	9230. 5	10386. 1	15767. 1

TABLE 6

Matching of protein biomarkers of ***Escherichia coli*** protein extracts with molecular weights of protein sequences available in SWISS PROT.

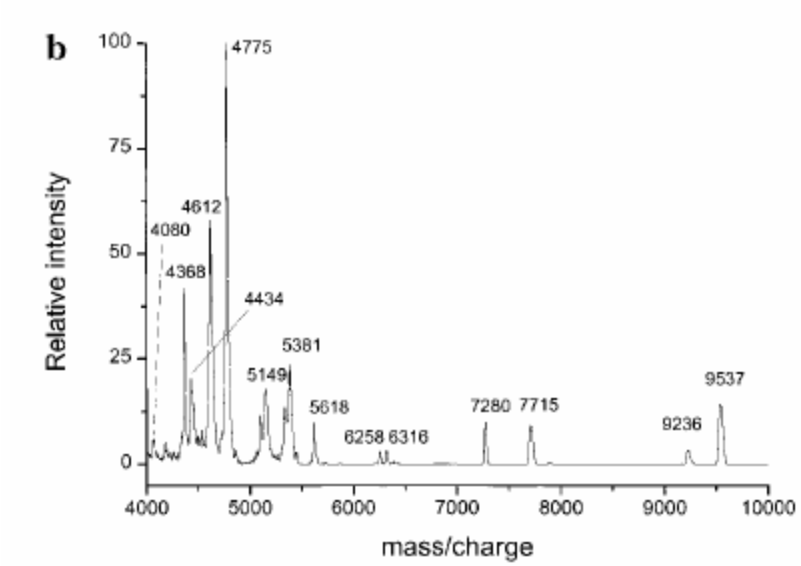
Organism	K1	K2
Escherichia coli	127	**30**
Anabaena sp	32	12
Mycobacterium tuberculosis	26	5
Bacillus subtilis	27	10
Helicobacter pylori	18	4
Pseudomonas aeruginosa	28	12
Salmonella typhi	52	**21**
Yersinia pestis	29	11
Vibrio vulnificus	36	15
Ralstonia solanacearum	23	12
Bacillus anthracis	27	5
Streptococcus pyogenes	36	8
Staphylococcus aureus	34	6
Bacillus cereus	27	9

RESULTS**:** ***Escherichia coli*** **(Escherichia coli ranks Ist as seen in column K2 for TABLE above)**

2. 5 CASE 5.

CHARACTERIZATION of *Escherichia coli* (ATCC No. 11775)

- Proteinous Biomarkers of ***E. coli samples*** are detected by MALDI TOF Mass Spectrmetry[13].
- Kompact MALDI 4 TOF instrument (Kratos Analytical Instruments, U. K.) [13].



E. coli (32 h growth time), matrix CHCA (see Experimental Section for more details)[13].

TABLE 7

Matching of protein biomarkers of ***Escherichia coli*** protein extracts with molecular weights of protein sequences from SWISS PROT.

Organism	K1	K2
Staphylococcus aureus	9	3
Escherichia coli	**43**	**14**
Bacillus subtilis	5	2
Bacillus cereus	11	4
Pseudomonas putida	12	3
Yersinia pestis	14	3
Staphylococcus epidermidis	10	4
Streptococcus pneumoniae.	17	5
Mycobacterium leprae	4	2
Neisseria meningitidis	14	3
Synechococcus sp	11	3
Pseudomonas aeruginosa	9	6

RESULTS: Escherichia coli (As seen in column K2 of the TABLE above, Escherichia coli ranks Ist)

3. CHARACTERIZTION OF BACTERIA BY THE USE OF MASS TABLES

For *Escherichia coli, Bacillus subtilis, C. Freundii, and B. megaterium . These bacteria can be found in the ambient environment**•MALDI experiments were performed on a Bruker REFLEX III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Framingham, MA). Desorbed proteins ions were produced with a 337 nm pulsed nitrogen laser and detected in the linear mode of operation.[10-12] . A total of 478 individual masses were detected by LC/offline-MALDI over the entire incubation period studied. Protein masses obtained for a particular growth time were about 20-25 % of those obtained for the entire incubation period.[10-12] Also the common masses obtained for at least any two growth times were about 20% of those obtained over the entire incubation period reported (MALDI TOF OR LC-ESI MS)[17]

3.1 CASE 6.

CHARACTERIZATION OF *Bacillus subtilis*

Proteinous Biomarkers of ***B. subtilis,*** **samples** are detected by MALDI TOF Mass Spectrmetry[13].
•Kompact MALDI 4 TOF instrument (Kratos Analytical Instruments, U. K.)[13].
•Mass specra used is same as reported in case 2.

Results : Very poor results. (Only two matches found from proteins found in mass tables corresponding to 11Hr, growth time for B. subtilis, only one match found corresponding to 16Hr. Only two matches found in 36Hr. No match found corresponding to any other growth period.)

3. 2 CASE 6A(By the Use of mass tables)•

Proteinous Biomarkers of *B. megaterium* **samples *are*** detected by On-Line LC/ESI-MS[17] of the protein extracts. Same mass tables are used to see how B. megaterium, interferes with ID of ***Bacillus subtilis.***

•HP 1100 MSD quadrupole mass spectrometer (Agilent). The *m*/*z* range from 500 to 3000[17], was scanned in 1. 90 s, and the ions were detected with a high-energy dynode detector[17]. One matche found in protein mass tables corresponding to 24Hr, growth time for Bacillus subtilis . Only one match found in mass tables corresponding to 36Hr, Only one match found in 48Hr, No molecular weight match found corresponding to mass tables for other growth times for Bacillus megaterium.

RESULTS: Over all poor results for characterization of *Bacillus subtilis* by the use of mass tables

3. 3 CASE 7 CHARACTERIZATION OF *Bacillus subtilis*

This uses only those protein masses for BG detected through MALDI TOF[10-12], which are common to at least two different variables in growth time for bacillus subtilis[12]. The mass spectra used are the one reported in case 2 above.

•**Mass Tables used** (Common between two variable time of growth for ***Bacillus subtilis***)---Daltons

2163 2400 2594 2597 2682 2697 2730 2761 2847 3002 3008

3155 3177 3284 3333 3476 3678 3833 4040 4524 4882 5084 5712 7296 7319 7326 2580 3356 3715 4116 7312 7341 2563 3276 3934 3949 4681 4808 5350 6064 7353 2220 2861 3747 4009 4312 7268 2318 3544 3730 11304

Results: No match found

3. 3 CASE 7a(By the Use of mass tables)

This uses only those protein masses for BG detected through MALDI TOF[10-12], which are common to at least two different variables in growth time for bacillus subtilis. 112 Proteinous Biomarkers of ***B. megaterium*** **samples *are*** detected by On-Line LC/ESI-MS[17] of the protein extracts. Same mass tables are used to see how B. megaterium, interferes with ID of ***Bacillus subtilis***•HP 1100 MSD quadrupole mass spectrometer (Agilent). The *m*/*z* range from 50 to 3000[17], was scanned in 1.90 s, and the ions were detected with a high-energy dynode detector[17].

Results: No match found; as seen above, this supports poor results for characterization of *B.subtilis*.

The results from two cases shows that, for the experimental mass spectra used, mass tables[10]for *Bacillus subtilis*, do not show good results for the known samples of *Bacillus subtilis.*

4. DISRUPTION OF BACTERIA SPORES

Preliminary experiments have been conducted in the BL-1 laboratory, for the comparison of technologies for the disruption of BG spores by the use of sonication or/and beads. The results showed more protein like peaks from the protein extracts, when beads were used for the disruption of quantities of BG spores. Further details are given in ECBC Tech. report. (unpublished). Some experimental details have been given under the experimental section

CONCLUSIONS

It is clear from various examples above that the use of the model system for identification of various bacteria, give better results than the use of mass tables, generated so for. [10-12,17]. In some cases as shown above, the use of statistical correction gives very poor results. But the use of this model system gave very good results for the characterization of those bacteria. In some case as seen above, the mere use of statistical correction (Significance testing), gives poor results. The significance testing used in conjunction with this model gives better results (Small proteome size). So this supports that method for identification may be used in conjunction with experimentally obtained mass tables for various bacteria. In some cases, we may also have to use the judgment based on biological agents of interest to Military. In situations of biological threat it is desired that we should have all the options available for identification of the biological agents. Although the experimental protein mass tables, as well as protein sequences for various bacteria, available in SWISS prot may be used in conjunction with each other for Bio-sample ID, meeting the time requirements, in some cases more information such as identity of protein may be required. An effort may

be made to experimentally find, protein sequences for large number protein biomarkers, by conducting the tryptic digestion and ESI-MS/MS for the protein extracts of various biological samples. . But in case further protein identification is required for the biological sample, detection and identification, the materiel used (BSPS and MS) should have the capability to conduct MS/MS after the tryptic digestion of the biological protein extracts. This may meet the demands in the field situations in terms of the point detection and identification of biological material. For this purpose we should have sufficient protein fragment data for the biological agents, so that it may be available, if needed. Relational databases management system may be used for storage as well as for the purpose of making decisions for the mass spectral data. This may be coupled with other commercially available software, for LC-ESI mass spectrometry. This approach has been discussed in the ECBC technical report (under publication).

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